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抄録：水中の化学物質による光合成への影響の指標としてそう類を用い，生理学的状態を，迅速試験法として開発したけい光測定により評価した。環境化学物質の代謝全体への影響は成長曲線によりモニターされるが，この目的にもけい光測定は有用である。尿素誘導体，モニュロン(I)，ジウロンおよびモノリニュロンは光合成を強く阻害し，クロルプロファムおよびペンタクロロフェノールは中程度の阻害を示す。重金属(Pb, Cd, Hg)は10-3mol/lの濃度で成長を阻害する。Hgによる成長阻害は初期(100時間まで)には最大であるが，数日後には阻害は消失する。重金属は光合成活性を低下させない。Iは10-6mol/lの濃度で成長を阻害し，IとCdとの間の相乗作用はみられない
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The *In-Vivo* Fluorescence of Chlorella Fusca as a Biological Test for the Inhibition of Photosynthesis

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Algae are used as indicators to determine the effect on photosynthesis caused by chemical substances introduced into waters. Their physiological condition is gauged by a fluorescence measurement, which has been developed into a rapid test. Impacts of environmental chemicals on the overall metabolism are preferably monitored by means of growth curves, but even for this purpose fluorometric determination of cell quantities have advantages over less rapid methods of analysis.

INTRODUCTION

Biological damage due to environmental chemicals can be estimated by means of toxicological tests on selected organisms. A catalogue of most current experimental work is given in Refs. 1 and 2. For the primary production process in aquatic systems, algae are usually proposed as test organisms.[3,4] The impact of a substance on the rate of cell multiplication can be used as a measure of its toxicity and is frequently determined experimentally by means of the turbidity of the culture, or by metabolic parameters such as O_2-production or 14-CO_2-incorporation.[5,6]

The effect on photosynthetic activity may be observed significantly faster by means of the fluorescent properties of the algae.[7] Generally,

it may be said that damaged algae re-emit a higher amount of absorbed light energy in the form of fluorescence than do active algae, because they cannot utilise the energy in the photosynthetic process. If a sample of algae in contact with an environmental chemical shows higher fluorescence than usual, this is an indication of the toxicity of the substance. Arndt[8] describes an application of this effect for air pollution monitoring.

Fluorescence measurements can also be applied to have determination of growth rates of cultures. The relative fluorescence intensity and the amount of chlorophyll are closely correlated in CMU-treated samples. (For abbreviations see Table I). Fluorescence measurements are highly sensitive.

TABLE I
List of test-substances

CMU (Monuron)	3-(4-Chlorophenyl)-1, 1-dimethylurea
DCMU (Diuron)	3-(3, 4-Dichlorophenyl)-1, 1-dimethylurea
Monolinuron	3-(4-Chlorophenyl)-1-methoxy-1-methyl-urea
Chlorpropham	Isopropyl-N(3-chlorophenyl)-carbamate
Pentachlorphenol	
Dieldrin	1, 2, 3, 4, 10, 10-Hexachloro-6, 7-epoxy-1, 4, 4a, 5, 6, 7, 8, 8a-octahydro-1, 4-endo-5, 8-exodimethanonaphtalene
Chlorthiamid	2, 6-Dichlorothiobenzamide
Biphenyl	
2, 4 DDT	1, 1, 1-Trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)-ethane
Dichlorobiphenyl	4, 4'-Dichlorobiphenyl
Cortisone acetate	21-Acetoxy-4-pregnen-17α-ol-3, 11, 20-trion
Lindan	γ-1, 2, 3, 4, 5, 6-Hexachlorocyclohexane
Dichlorophenoxyacetic acid	2, 4-Dichlorophenoxy-acetic acid
Maleic anhydride	
Carbaryl	1-Naphtyl-N-methylcarbamate
Pb	Lead(II)acetate
Cd	Cadmiumnitrate
Hg	Mercury(II)chloride

MATERIALS AND METHODS

The cultures of Chlorella fusca were not synchronised, but subjected to an artificial (8/16 h) day–night rhythm. Illumination was provided by a combination of 20W/15 (white) and 20W/77 (fluora) lamps in the ratio 1:2. The light intensity was of the order of 10 kLux. The culture tanks were aerated and maintained in a water bath at 30°C. The nutrient solution was used as described by Kuhl.[9] Water insoluble test substances were first dissolved in acetone, and then diluted with double-distilled water to the desired concentration. The proportion of acetone in the sample was always less than 1‰, which was without significant influence on the algae.

Figure 1 curve a, shows the time dependence of the fluorescence intensity of an algae suspension under constant illumination after adaptation to dark. The fluorescence output of the chlorophyll molecules, which interact with the electron transport chain of the photosynthetic mechanism, increases sharply, reaches a maximum and then decreases slowly to a final value. This effect was observed by Kautsky as early as 1943.[10] Algae with an inhibited electron transport chain very quickly reach a higher, constant level (Curve b). The difference in fluorescence intensity is a measure of the energy

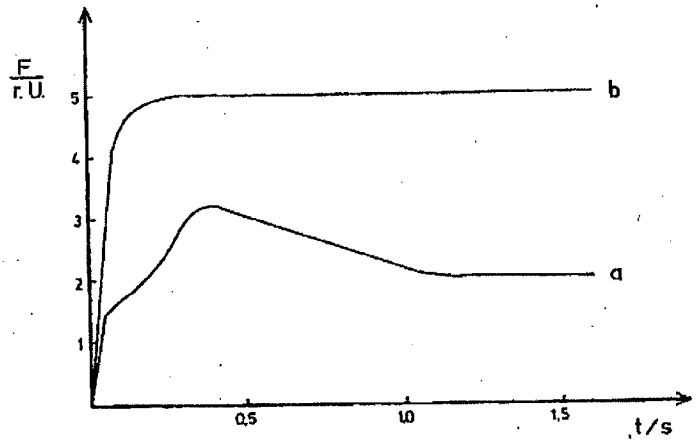

FIGURE 1 Kautsky curve of Chlorella fusca (a) without (b) with CMU additive.

being utilised in photosynthesis. Furthermore, the Kautsky effect, that is the increase of the peak fluorescence intensity above the final value, can equally well be used as a measure of the photosynthetic activity of the algae. Dead algae display no such effect.

In order to arrive at a convenient method of measurement, the fluorescence measurements are performed with a specially developed flashlight fluorometer, which is battery operated and therefore suitable for field experiments.[11] Excitation results from a wavelength of 435 nm (absorption maximum of chlorophyll a), and fluorescence emission is measured at 682 nm. The light path in the equipment is vertical to eliminate errors due to sedimentation of algae.[12]

To perform a measurement, 1 ml algae suspension of a 3 days old culture is diluted in the measuring vessel with 24 ml nutrient solution. The algae concentration should lie in the region of 10^7 cells/ml. Prior to the measurement the sample is kept in the darkness for 5 minutes by leaving it in the light proof fluorometer. After this period the photosynthetic activity is almost zero. The sample is then exposed to a light flash. The fluorescent intensity, integrated over a time interval of 500 ms yields a value F_1 which represents the maximum of the Kautsky curve (Figure 2). This light flash also stimulates the algae to greater photosynthetic activity. A further light flash after an interval of one minute, thus produces a lower value of fluororescence, F_2. Further flashes produce a series of approximately equal values, which represent the plateau of the Kautsky curve.

If a toxic substance is added to the sample the fluorescence values F'_1 and F'_2 are observed to be higher after the five minutes darkness and they differ by less than do F_1 and F_2.

In order to estimate the toxic effect quantitatively, the following parameters are defined:

$$V = \frac{F'_2 - F_2}{F'_2} \times 100\%$$

$$K = \frac{\dfrac{F'_1 - F'_2}{F'_1}}{\dfrac{F_1 - F_2}{F_1}} \times 100\%$$

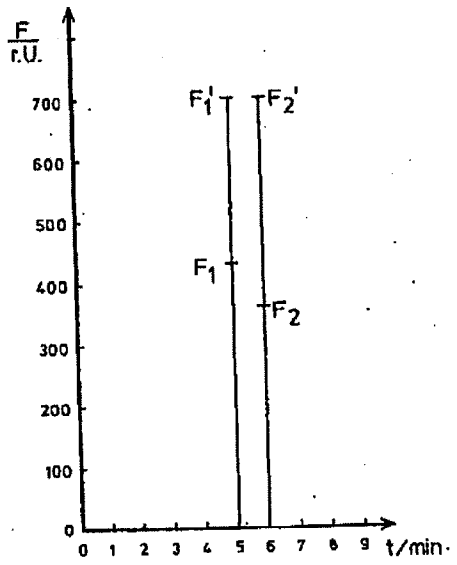

FIGURE 2 Relative fluorescence intensities measured with flash light fluorometer. Uncontaminated sample: F_1, F_2. Contaminated sample F_1', F_2'. Darkness adaptation interval 5 min in both cases.

In accordance with the above expressions, the toxic effect of a substance is greater, the larger the value of V and the smaller the value of K. Normalisation results in the values being independent of algae concentration. The value of K additionally takes into account possible variations in activity of the algae before the addition of the toxin (the denominator is a measure for this activity). The reliability of the values of F_2 and F_2' may be increased by repeated flashing of the same sample and taking an average value. This leads to the value of V being more reproducible than the K value.

If long-term effects are observed, growth curves are monitored, which reflect the overall metabolic activity of the algae. The toxins are added to the nutrient solution. One cannot eliminate the time-consuming observations of the samples over the life span of the culture, but even here fluorescence methods have advantages over less rapid methods of analysis. The relative fluorescence intensities of algae samples are proportional to the mass of chlorophyll present

and hence are closely correlated to the number of algae present, provided that the fluorescence signal is not affected by photosynthesis. To stop photosynthesis CMU (10^{-3} mol/l) is added to the samples, prior to the fluorescence measurements. CMU inhibits the electron transport chain in the photosynthetic membranes of algae.

RESULTS AND DISCUSSION

Figure 3 shows photosynthetic activity (expressed in terms of K) as a function of various CMU concentrations, 50% reduction is reached at a concentration of approx. 10^{-6} mol/l. Thus this rapid test is only about one order of magnitude less sensitive against CMU than electron transport inhibition tests on isolated chloroplasts.[13]

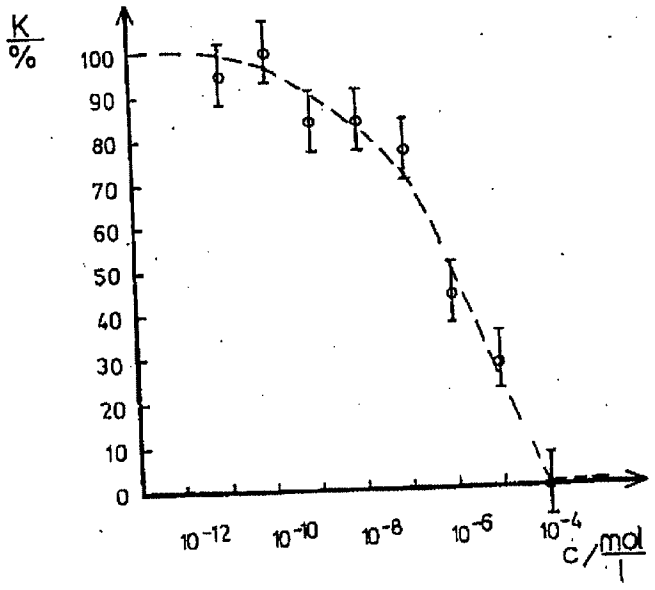

FIGURE 3 Photosynthetic activity of Chlorella fusca as a function of CMU concentration. Bars depict the limits of absolute errors.

In Figure 4, the effect of 15 selected environmental chemicals on the photosynthetic activity of Chlorella fusca is shown. The concentration of the toxins in each case is 4×10^{-5} mol/l. The shaded columns represent K values, the unshaded V values.

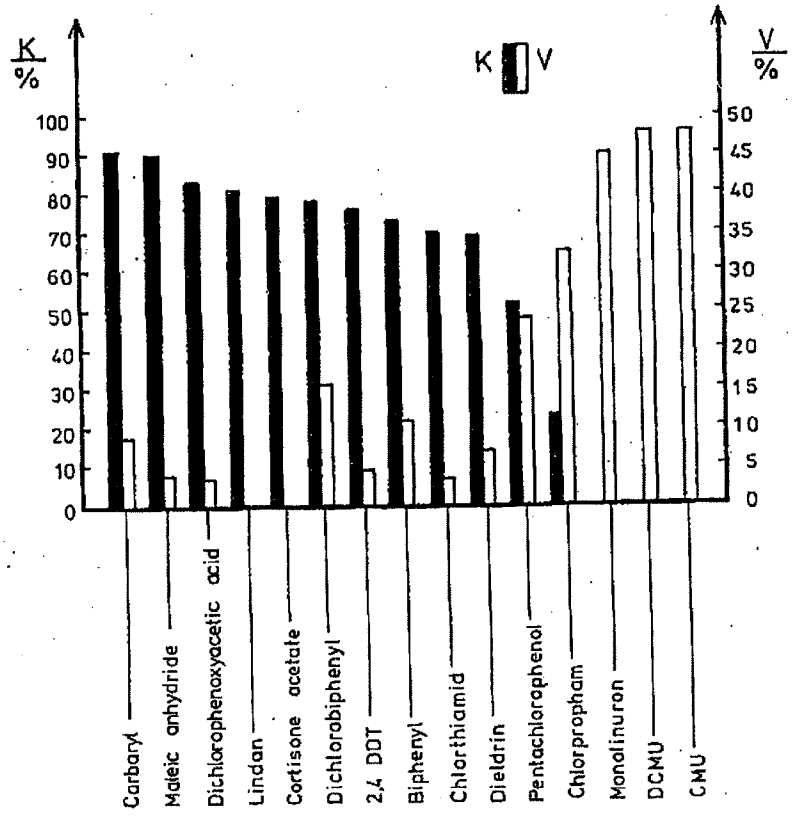

FIGURE 4 Influence of some environmental chemicals on photosynthetic activity of Chlorella fusca. Severe inhibition indicated by low K-values, and high V values.

The urea derivatives CMU (monuron), DCMU (diuron) and monolinuron have the greatest effect. These herbicides inhibit the electron transfer in photosystem II. The soil herbicide chlorpropham, which also influences photosynthesis, has a somewhat milder effect.

The next most toxic substance is the bactericide, herbicide and fungicide pentachlorophenol, which as a cell poison interferes with oxidative phosphorylation and enzyme supply. The minimal effect of another herbicide, chlorothiamid, is striking. This herbicide is, however, first transformed into the very effective substance dichlorbenil in the soil and in the treated plant. This activation appears not to occur in the algae within the 5 minutes period.

The other substances administered cause only very slight damage to the photosynthetic mechanism, and their ranking of effectiveness by K values is somewhat different to their ranking by V values. The deviations however are within the limits of error.

The rapid test in this form thus provides mainly a test for photosynthesis inhibitors. If a test yields negative results we may state that the substance under investigation does not directly damage the photosynthic system. However, there may still be adverse effects on other parts of the metabolism.

The effect of heavy metals was not discernible within a few minutes (corresponding to the darkness period), hence rates of growth were determined. In Figure 5, the growth curves are shown for cultures which were grown in nutrient solutions containing heavy metals. From this it can be seen, that Hg has the greatest initial inhibiting effect, but that this effect diminishes after a few days. Heavy metal concentrations at which observable inhibition of growth occurs, are so high, however, that Chlorella fusca is not suitable as test organism for these substances.

As mentioned before, CMU has to be added to the algae samples prior to the measurements, in order to assure the fluorescence intensities being independent of photosynthetic activity. Additional estimations of the fluorescence intensities without adding CMU yielded considerable lower values in all cases. This shows that the photosynthetic activity was not reduced by the presence of heavy metals. The impairments occur elsewhere, and do not initially affect the photosynthetic mechanism. Photosynthesis is inhibited only after the cell damage reaches such levels that cell division is no longer possible.

Figure 6 shows the growth curve of a culture whose nutrient solution has been contaminated with incremental CMU concentrations. The initial culture was 3 days old. A fluorescence value of 1000 $r.U.$ corresponds to an algal concentration of approx.

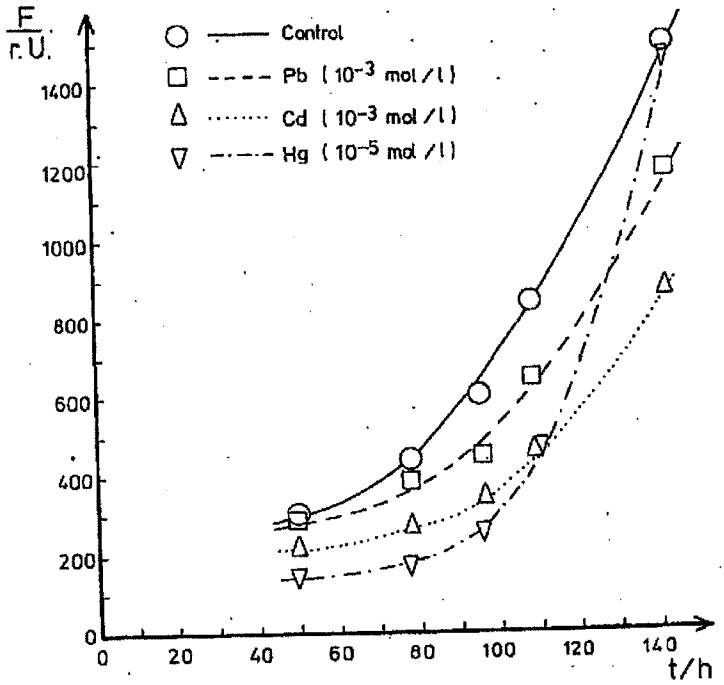

FIGURE 5 Influence of heavy metals on algae growth.

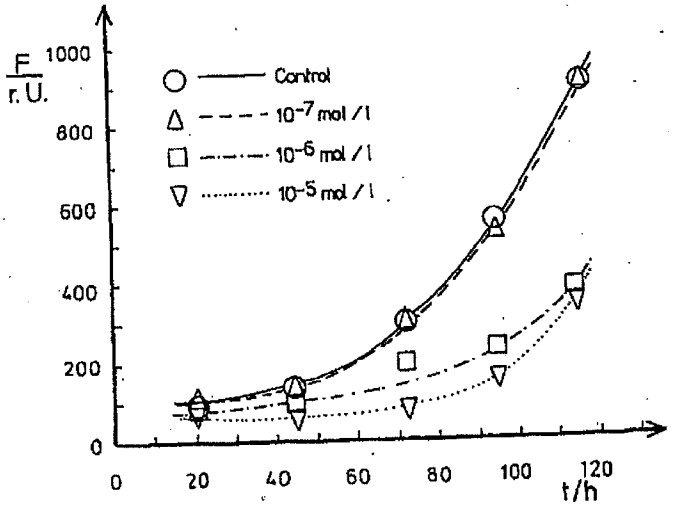

FIGURE 6 Growth curves under the action of incremental CMU concentration.

40×10^6 algae/ml. Only a CMU concentration of 10^{-6} mol/l results in a noticeably diminished growth rate. Likewise the rapid test gave a result of $K = 50\%$ at this concentration e.g. in this case the time consuming estimation of growth curves does not give higher sensitivity.

In a further series of experiments, synergistic effects between simultaneous action of herbicides and heavy metals were investigated. It is apparent that the total effect is determined by the more toxic substance alone. Figure 7 shows as an example growth curves with CMU and Cadmium admixtures. The result shows again that the impairments affect different areas of the metabolism.

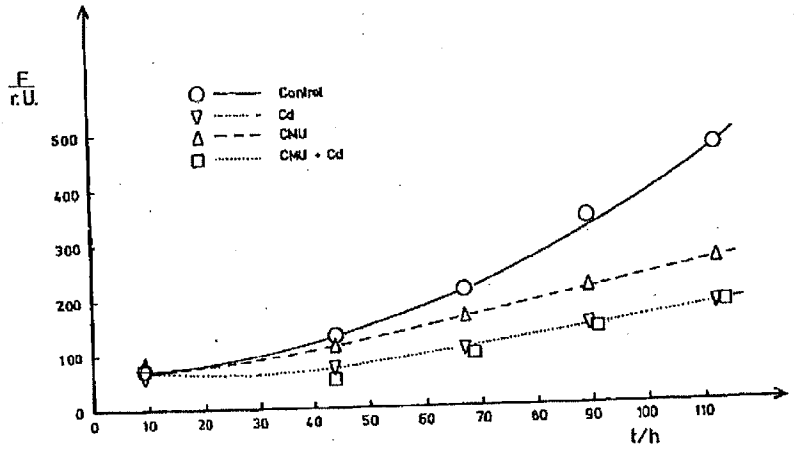

FIGURE 7 Growth curves of Chlorella fusca under the action of CMU, Cd and CMU+Cd.

Acknowledgements

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